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Distinct chromosomal profiles in metastasizing and non-metastasizing colorectal carcinomas

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Abstract. *Background*: The prognosis of colorectal cancer patients is to a considerable extent determined by the metastatic potency of the primary tumor. However, despite the fact that liver metastases are the leading cause of death for cancer patients, the molecular basis still remains poorly understood and independent prognostic markers have not been established. *Materials and methods*: Comparative genomic hybridization (CGH) was used to screen colorectal carcinomas without distant metastases ($n = 18$) and carcinomas synchronously metastatic to the liver ($n = 18$). We aimed to detect distinct chromosomal aberrations indicating a metastatic phenotype. *Results and discussion*: Metastatic tumors exhibited a significantly ($P = 0.03$) higher ANCA value (13.8) if compared with non-metastatic cancers (10.0). Furthermore, we observed that losses of chromosomal regions 1p32-ter and 9q33-ter were present at much higher frequencies in metastatic than in non-metastatic cancers, respectively ($P = 0.02$ and 0.04). *Conclusion*: These data indicate that metastatic tumors may be separated from non-metastatic colorectal cancers based on their genomic profile.

Keywords: Colorectal cancer, comparative genomic hybridization, liver metastases, predictive marker, prognosis

Abbreviations: UICC, International Union against Cancer; CGH, comparative genomic hybridization; ANCA, average number of chromosomal copy alterations.

1. Introduction

The stepwise progression of colorectal carcinomas is accompanied by gains of chromosomes 7, 8q, 13 and 20, as well as losses of chromosomes 4, 8p and 18q [9,20,28,36]. In a previous study we demonstrated that gains of chromosome 8q23-24 are associated with lymph node positivity in non-metastatic colorectal carcinomas [16]. Whereas this aberration was detected in the vast majority of lymph node positive tumors, it was only rarely present in lymph node negative carcinomas suggesting that genes located at 8q23-24 might favor the development of lymphatic metastases in colorectal cancers. Regarding metastatic disease, i.e. tumors with systemic spread, several investigators evaluated the underlying genomic changes of advanced colorectal cancers. But the results remain contradictory, and a chromosomal aberration based metastatic genotype has not been established [2–4,6,10,24,25,31,33].

From the clinical point of view, a significant number of colorectal cancer patients develop distant metastases, preferably to the liver, even though approximately 60% to 70% of these patients will undergo potential curative surgery at the time of cancer diagnosis [39]. Therefore, adjuvant chemotherapy is widely considered as the gold standard for patients with UICC stage III colon cancer, who are at high risk of recurrence [1,8]. Nevertheless, even stage II tumors have the potential to form distant metastases, and approximately 20% of these patients die of recurrent disease [29]. Prospective randomized trials have therefore been initiated to evaluate the potential benefit of adjuvant

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chemotherapy for stage II colon cancer patients. Accordingly, accurate predictive biomarkers would help to establish an individualized, metastatic phenotype-based therapy [18].

The aim of the present study was to investigate distinct patterns of copy number alterations in advanced colorectal cancers. We therefore used comparative genomic hybridization (CGH) to determine genomic differences between non-metastatic colorectal cancers (mainly UICC III) and cancers synchronously metastatic to the liver (UICC stage IV) in an attempt to identify chromosomal aberrations that might serve as genetic markers for the risk of liver metastases.

2. Materials and methods

2.1. Materials

In the present study, we prospectively collected surgical specimens from 36 patients diagnosed with a colorectal cancer between 2000 and 2002. Only fresh frozen tumor samples with a tumor cell content of at least 70% (established on hematoxylin–eosin-stained tissue sections) were analyzed. The histopathological classification was based on the WHO histological typing of colorectal cancers [38]. The clinical data are summarized in Table 1. All tumors were adenocarcinomas and have been classified as either non-metastatic carcinomas (group 1; pT2-4 pN0-2 M0; $n = 18$) or as cancers with hepatic metastases (group 2; pT2-4 pN0-2 M1; $n = 18$). We only selected cancer patients exhibiting liver metastases synchronously, defined as diagnosed within 6 months following diagnosis of the primary tumor.

2.2. Comparative genomic hybridization

CGH experiments and analysis were performed as previously described [15]. Briefly, CGH was performed on normal, sex-matched metaphase chromosomes prepared according to standard procedures following the criteria by du Manoir and colleagues [13]. Normal DNA was labeled in a standard nick-translation reaction substituting dTTP with digoxigenin-12-dUTP (Roche; Mannheim, Germany). Tumor DNA was extracted using a commercially available DNA-isolation KIT from Qiagen (Hilden, Germany) and labeled by substituting dTTP with biotin-16-dUTP (Roche; Mannheim, Germany).

For CGH, 300 ng of normal digoxigenin-labeled and 300 ng of biotin-labeled tumor DNA were ethanol precipitated in the presence of 30 μg of the Cot-1 fraction of human DNA (Roche; Mannheim, Germany). The probe DNA was dried and resuspended in 10 μl of hybridization solution (50% formamide, 2$\times$ SSC, 10% dextran sulfate), denatured (5 minutes at 75°C), and preannealed for 1 hour at 37°C. The normal metaphase chromosomes were denatured separately (70% formamide, 2$\times$ SSC) for 2 minutes at 75°C. Hybridization took place under a coverslip for 3 days at 37°C. Posthybridization steps were performed as described in detail elsewhere [15]. Biotin-labeled tumor sequences were detected with FITC conjugated to avidin (Vector laboratories; Burlingame, CA), and the digoxigenin-labeled reference DNA was visualized with antidigoxigenin Fab fragments conjugated to rhodamine (Roche; Mannheim, Germany). The chromosomes were counterstained with 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories; Burlingame, CA) and embedded in an antifade solution containing paraphenylenediamine (Sigma; St. Louis, USA).

Gray-level images were acquired for each fluorochrome using a CCD camera (Sensys, Photometrics, Munich, Germany) coupled to an epifluorescence microscope (Axiovert 25, Zeiss, Jena, Germany) using sequential exposure through fluorochrome specific filters. For automated karyotyping and analysis a software package was used (Quips Karyotyping/CGH; Vysis; Downer's Grove, USA). At least 12–15 metaphases have been evaluated according to the guidelines suggested in the ISCN 1995 [30]. The karyograms (Figs 1 and 2) summarize the individual CGH experiments. The lines to the left of the chromosomal ideograms indicate chromosomal losses (ratio of 0.8), the lines to the right chromosomal gains (ratio of 1.2). Bold lines indicate high-level copy number increases, exceeding a threshold of 1.5 (amplifications).

Genomic instability was estimated as the average number of copy alterations (ANCA), which is deduced by dividing the total number of chromosomal copy alterations in a karyogram (see Figs 1 and 2) by the number of tumors analyzed (for details see [37]).

2.3. Data analysis

We first compared the average number of chromosomal aberrations (ANCA values) of the non-metastatic and the metastatic tumors with a two-sided t-test for independent samples. In addition, Fisher's exact test was used to determine the potential significant influence of

Table 1
Clinical data of 36 patients with colorectal cancer

	Age (yrs)	Sex	TNM staging	UICC stage	Grading	Localization
Group 1						
28	63	F	pT2 pN0 (0/23) M0	I	2	Ascending colon
27	54	M	pT3 pN0 (0/25) M0	II	2	Cecum
21	60	F	pT3 pN0 (0/22) M0	II	2	Rectum
20	53	M	pT3 pN0 (0/14) M0	II	2	Sigmoid colon
19	67	M	pT3 pN0 (0/26) M0	II	2	Sigmoid colon
23	73	M	pT2 pN1 (2/16) M0	III	2	Sigmoid colon
25	81	M	pT2 pN1 (1/21) M0	III	2	Sigmoid colon
31	52	M	pT3 pN1 (1/33) M0	III	2	Ascending colon
35	73	M	pT3 pN1 (1/32) M0	III	2	Cecum
32	54	M	pT3 pN2 (19/21) M0	III	2	Rectum
24	71	M	pT3 pN2 (21/55) M0	III	2	Ascending colon
30	60	M	pT3 pN2 (6/13) M0	III	2	Rectum
29	66	F	pT3 pN2 (5/21) M0	III	2	Ascending colon
34	68	F	pT3 pN2 (14/15) M0	III	2	Rectum
33	65	F	pT3 pN2 (12/12) M0	III	2	Ascending colon
26	71	M	pT4 pN2 (9/19) M0	III	2	Cecum
36	65	M	pT4 pN2 (4/22) M0	III	2	Rectum
22	69	F	pT4 pN2 (9/21) M0	III	2	Cecum
Group 2						
18	63	M	pT2 pN0 (0/25) M1	IV	2	Rectum
7	67	F	pT3 pN0 (0/19) M1	IV	2	Descending colon
2	65	M	pT3 pN0 (0/10) M1	IV	2	Descending colon
5	66	M	pT3 pN0 (0/17) M1	IV	2	Sigmoid colon
16	60	M	pT3 pN0 (0/17) M1	IV	2	Rectum
15	68	M	pT2 pN1 (1/20) M1	IV	2	Rectum
8	49	F	pT2 pN2 (5/21) M1	IV	2	Rectum
13	65	M	pT3 pN1 (3/19) M1	IV	2	Rectum
12	53	M	pT3 pN1 (3/21) M1	IV	2	Rectum
6	78	M	pT3 pN2 (16/27) M1	IV	2	Sigmoid colon
11	78	F	pT3 pN2 (20/24) M1	IV	2	Transverse colon
3	61	F	pT3 pN2 (22/40) M1	IV	2	Ascending colon
14	64	M	pT3 pN2 (6/17) M1	IV	2	Rectum
10	78	F	pT3 pN2 (4/30) M1	IV	2	Ascending colon
17	60	M	pT3 pN2 (18/21) M1	IV	2	Rectum
9	75	F	pT4 pN2 (21/26) M1	IV	2	Rectum
1	49	F	pT4 pN2 (29/30) M1	IV	2	Sigmoid colon
4	50	M	pT4 pN2 (9/23) M1	IV	2	Sigmoid colon

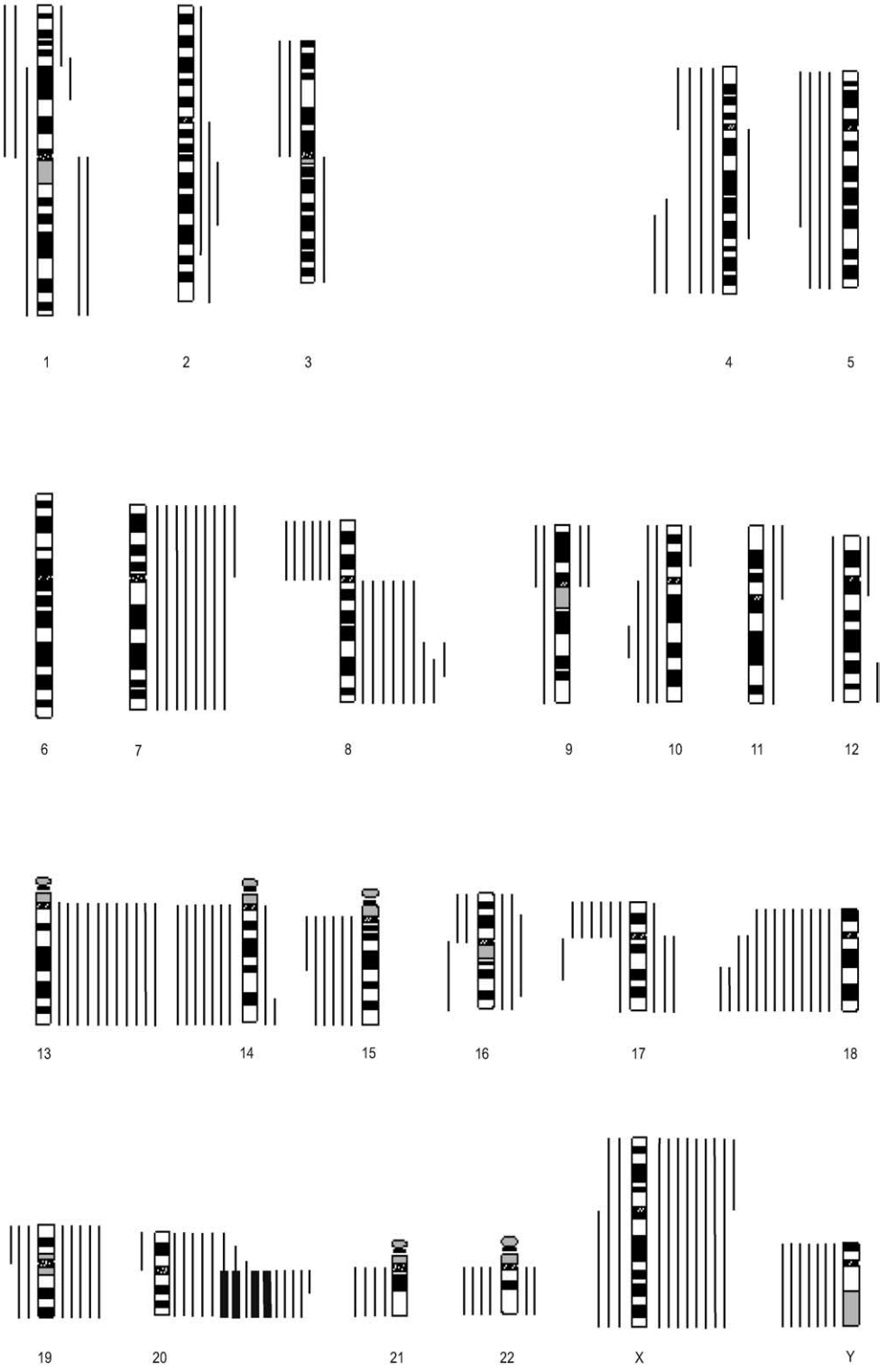

Fig. 1. Karyogram of chromosomal gains and losses in 18 colorectal carcinomas without liver metastases (group 1).

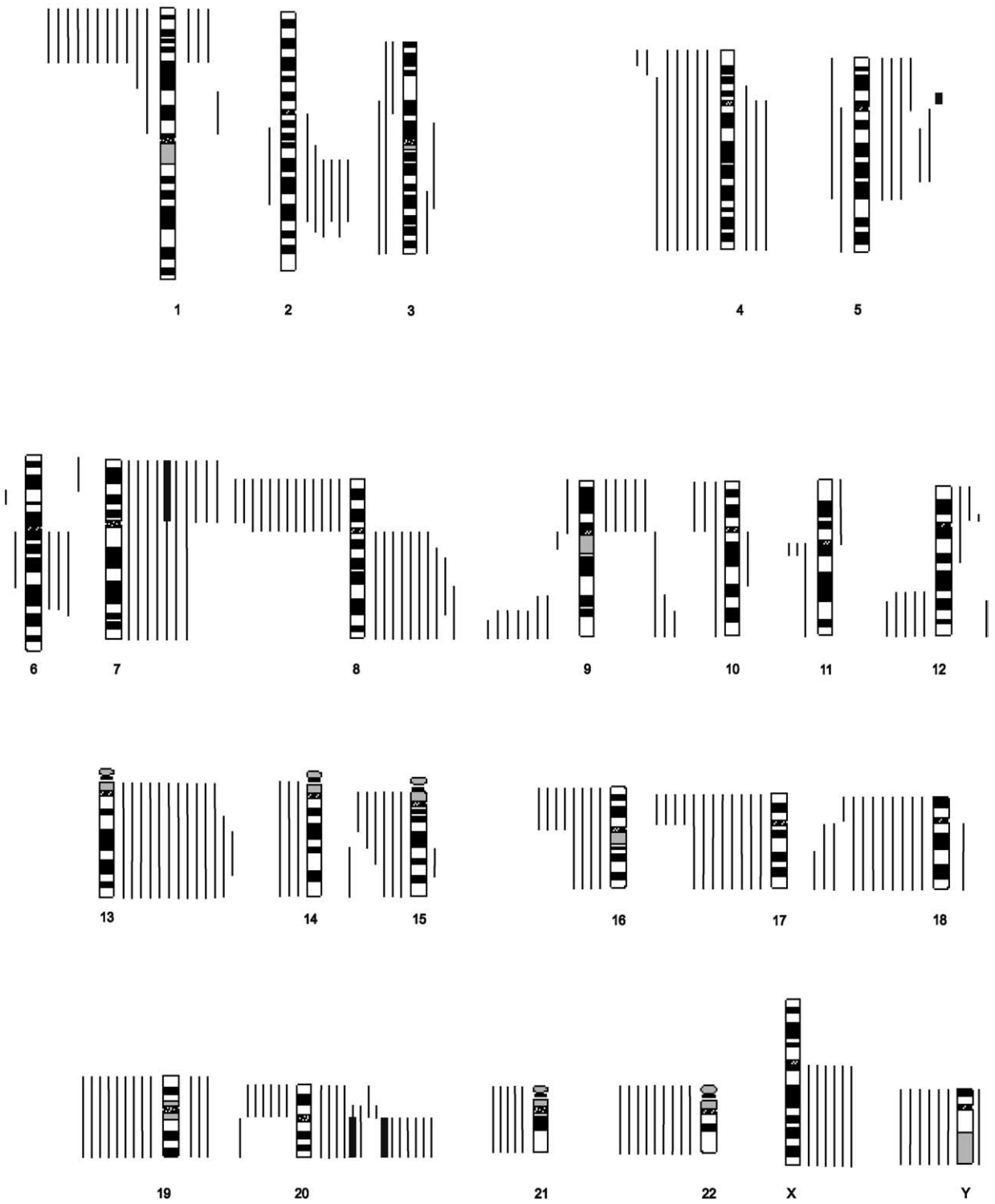

Fig. 2. Karyogram of chromosomal gains and losses in 18 colorectal carcinomas with synchronous liver metastases (group 2).

chromosomal gains and losses on the metastatic phenotype. Differences with a $P < 0.05$ were considered statistically significant.

3. Results

CGH was used to screen for copy number changes in 36 patients with colorectal cancer. The clinical data for all patients are presented in Table 1.

3.1. Colorectal carcinomas without liver metastases (group 1)

All 18 tumors displayed chromosomal imbalances. Overall, we detected 90 gains and 90 losses, resulting in an ANCA value of 10. Frequent gains affected regions on chromosomes 7 (50%), 8q (50%), 13 (61%), 20 (83%) and X (50%). Losses of chromosomal material frequently mapped to 8p (33%), 14 (39%), 15 (33%), 17p (33%) and 18q (72%). Amplifications localized exclusively to chromosome arm 20q. Figure 1 summarizes the chromosomal aberrations in the analyzed non-metastatic tumors.

3.2. Colorectal carcinomas with synchronous liver metastases (group 2)

In this group of carcinomas ($n = 18$), we observed an ANCA value of 13.8 (104 gains and 144 losses; Fig. 2). DNA gains affected regions of chromosomes 7 (56%), 8q (56%), 13 (72%) and 20 (83%). High-level copy number increases could be mapped to chromosomal band 5p13 and chromosome arms 7p and 20q. Decreased values were observed for chromosomal regions of 1p (61%), 4q (33%), 8p (67%), 9q (39%), 15 (39%), 16p (44%), 17p (67%), 17q (44%), 18q (67%), 19 (50%), 20p (33) and 22 (50%).

3.3. Comparison of non-metastatic and metastatic colorectal carcinomas

The metastatic tumors displayed a higher degree of chromosomal instability than the non-metastatic tumors, which is reflected by a mean ANCA value of 13.8 in group 2 and 9.9 in group 1, respectively ($P = 0.03$). Additionally, UICC stage IV cancers showed significantly more chromosomal losses ($P = 0.01$), whereas no significant difference could be detected when comparing the chromosomal gains in the two groups ($P = 0.33$). Furthermore, we identified two

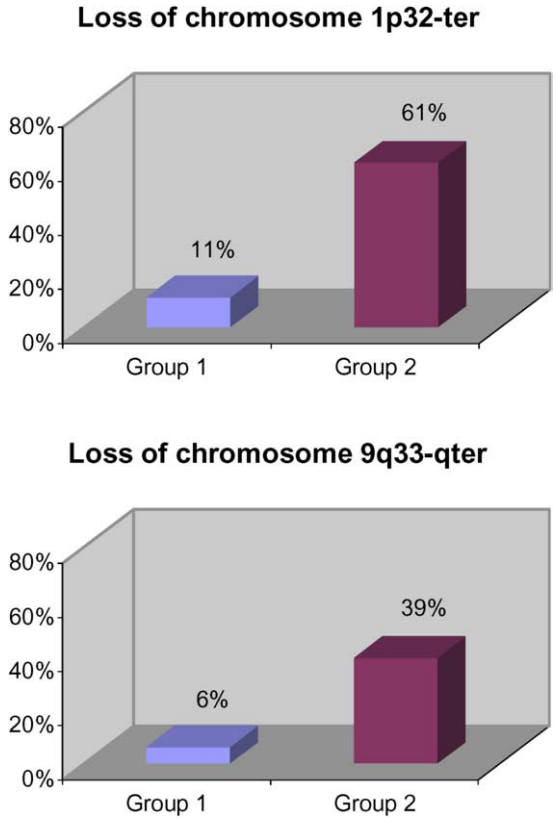

Fig. 3. Frequencies of sub-chromosomal imbalances in metastatic (group 2) and non-metastatic (group 1) colorectal cancers.

distinct chromosomal losses which were present at much higher frequencies in metastatic than in non-metastatic cancers, losses of 1p32-ter ($P = 0.02$) and 9q33-ter ($P = 0.04$), respectively (Fig. 3). Of note, chromosomal gains with higher frequencies in metastatic cancers could only be mapped to chromosome 5p ($P = 0.05$).

4. Discussion

Several investigators evaluated the genomic changes underlying metastases formation of colorectal cancers using CGH, but the results remain contradictory (recently reviewed in [11,19]). Analyzing primary Dukes' stage C and D carcinomas and corresponding metastases, Al-Mulla and colleagues found that gains of chromosome arms 17q and 6p were significantly associated with metastatic disease [3]. Nakao and colleagues reported that primary metastatic tumors showed higher frequencies of gains of 6q, 7q, 8q, 13q and 20q [31]. Some of these findings have

been particularly confirmed by other groups, looking at either primary tumors or metastases [4,10,33]. However, many authors stated that the primary tumors investigated neither showed identical nor totally different genomic aberrations when compared with the corresponding metastases. Usually, the metastases contained additional gains and losses [3,4,21,24,25]. This is supported by findings from Alcock and colleagues, who analyzed microdissected sub-regions from primary tumors and corresponding hepatic metastases. They reported that no two samples from one case were identical, although common changes like gains of X and 12q as well as losses of 8p, 16p, 9p, 1q, 18q and 10q were identified [2]. Interestingly, de Angelis and colleagues did not find any chromosomal aberration correlated with clinical stage [9].

Accordingly, defined chromosomal aberrations that clearly distinguish metastatic colorectal tumors from non-metastatic tumors remain to be established. However, it is important to note that some groups analyzed DNA from primary tumors, whereas others examined metastases. Since tumors with a potential metastatic phenotype should already be identified at the time of diagnosis, i.e. at the time a biopsy is taken, we favor to analyze the genomic features of the primary tumor. One could also argue that, depending on the time of resection, metastases could potentially have accumulated additional DNA changes. These aberrations would then not mirror the genomic features of the primary tumor, but instead just be the manifestation of a longer growth process in a selective environment.

In the present investigation, we used comparative genomic hybridization (CGH) to compare non-metastatic colorectal cancers with cancers synchronously metastatic to the liver. Our analysis revealed that both groups have certain chromosomal aberrations in common that have been previously identified, for example gains of 7, 8q, 13 and 20 and losses of 4, 8p, 14, 15, 17p and 18. In particular, since 62% of the UICC stage III cancers revealed gains of chromosome 8q23-24, we could confirm our previously reported result that lymph node positive cancers show high frequencies of this chromosomal aberration [16]. Additionally, this study demonstrates that metastatic tumors showed significantly more chromosomal losses than non-metastatic tumors ($P = 0.01$). Furthermore, we identified distinct chromosomal aberrations that were present at much higher frequencies in metastatic than in non-metastatic cancers, such as losses of 1p32-ter and 9q33-ter, respectively ($P = 0.02$ and 0.04). Most interestingly, these chromosomal losses were present in small metastatic tumors (pT2) as well as in locally advanced metastatic tumors (pT4), and in lymph node negative metastatic tumors as well as in lymph node positive metastatic tumors. Possibly, the incidence of these chromosomal aberrations reflects a highly aggressive metastatic genotype of colorectal cancers.

Even though it is well known that aberrations of chromosome 1p need to be interpreted with care [28], we think it is rather unlikely that they represent technical artifacts. Firstly, in the present investigation, these chromosomal aberrations have been predominantly detected in metastatic tumors, whereas loss of 1p and 9q was a rather rare event in non-metastatic tumors. Secondly, several studies have previously shown that chromosome arm 1p is commonly affected in colorectal adenocarcinomas. Deletions of 1p have been identified in colorectal adenocarcinomas using conventional cytogenetics, fluorescence *in situ* hybridization or loss of heterozygosity analyses, and the most frequently affected region has been shown to be 1p32-p36 [5,12, 14,17,27,34,35,40]. Furthermore, deletions in specific sub-regions of 1p have been associated with a poor prognosis [23,32].

In summary, this analysis identified distinct chromosomal losses that might separate metastatic from non-metastatic colorectal carcinomas, losses of chromosomal regions 1p32-ter and 9q33-ter, respectively. This indicates that it might be possible to establish reliable markers for prediction of the metastatic phenotype. Furthermore, one can speculate that tumor suppressor genes, which are predominantly inactivated via allelic loss, might be more important for the development of metastatic disease than oncogenes. *CDC2L1* [7], *CDC2L2* [26] and *TP73* [22] represent potential target genes on chromosome arm 1p. Obviously, these hypotheses need to be validated in larger prospective analyses.

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